EXAMINATION OF INDEPENDENTLY EVOLVING RESISTANCE IN 29 RESISTANT CELL LINES

Ph.D. Theses

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Introduction

Worldwide approximately 1 000 000 new breast cancer patients are diagnosed, and these lead to 370 000 deaths yearly. Breast cancer is the most frequent malignant tumor among women in Hungary, more than 2300 women die of breast cancer yearly. Despite the increasing frequency of breast cancer incidece, due to the improvements in systemic treatment and breast cancer screening campaigns the disease specific mortality decreased in most of the developed countries. Most important risk factors are age, gender, hormonal factors, and family anamnesis. However familiar aggregation of the disease is rare, only 10% of the patients have a germline mutation. The treatment is based on surgery, radiotherapy, chemotherapy, endocrine and targeted therapy.

Doxorubicin and paclitaxel have a central role in the chemotherapy of breast cancer patients. Chemotherapy resistance is a complex multifactorial problem, where several factors may act simultaneously, leading to failure of systemic treatment.

Doxorubicin is able to intercalate into the DNA. The DNA-bound doxorubicin inhibits topoisomerase II, leading to breaks in the DNA chain, and this induces apoptosis. Doxorubicin resistance is mediated by the increased activity of P-glycoprotein, lung resistance

protein and topoisomerase; by the altered expression of proteasome subunits and enzymes involved in antioxidant defenses and by the changed apoptotic pathways.

Paclitaxel stabilizes microtubules, preventing chromosome segregation in the anaphase of the cell cycle. Paclitaxel resistance is mediated by ABC transporters (MDR1), expression changes of tubulin isoforms and microtubule-associated proteins and mutations of the tubulin gene.

The efficacy of the treatment, and the emerging resistance mechanisms can efficiently be examined using *in vitro* models. Voskoglou-Nomikos et al. demonstrated, that the results of properly executed *in vitro* and xenograft experiments can predict the results of phase II trials.

In human malignant tumors intratumoral heterogeneity can be detected in terms of gene expression, cell morphology, metabolism, motility, proliferative, angiogenic, immunogenic and metastatic properties. During tumor growth new genetic alterations occur, and this also contributes to heterogeneity. The heterogeneous cell populations undergo selection during the treatment. This selection leads to the emergence of resistant tumor cell populations. As multiple mechanisms can lead to resistance, multiple cell populations with different resistance properties may survive the selection process, leading to heterogeneity in terms of resistance.

The development of acquired resistance was simulated by significantly increasing the number of parallel developed resistant cell lines. We assumed, that truly robust and relevant resistance mechanisms will emerge in multiple cell lines and a clinically relevant convergent pattern of resistance can be identified. Twenty-nine subpopulations of two breast cancer cell lines were separated and treated with increasing concentrations of doxorubicin and paclitaxel for 18 months. These cells were then investigated to explore whether known resistance mechanisms consistently emerge and therefore the same strategy could be used to identify novel mechanisms as well.

Aims

In this study I used more resistant cell lines deriving from the same cell line than in previous studies. The rationale behind this was the goal to better characterize the evolving resistance. I also investigated the multidrug resistance in this model. My questions were:

1 Can the examination of multiple resistant cell lines derived from the same cell line lead to the identification of multiple acquired resistance mechanisms described before for paclitaxel or for doxorubicin?

2 Does the resistance evolve in a consistent way in the resistant cell lines derived from the same cell lines, receiving the same drug?

3 Does multidrug resistance evolve, if only one drug is used during the treatment of cell lines?

Methods

Cell lines, treatment of cell lines, determination of cell proliferation

MCF-7 and MDA-MB-231 human breast cancer cell lines were treated with gradually increasing concentrations of paclitaxel and doxorubicin. The IC_{50} values of the cell lines were determined by MTT cell proliferation assay. A concentration range between 0.0001 times and 1000 times of the clinical concentration was used during the treatment period. Clinical dose was 0.02 μg/ml in case of doxorubicin, and 0.1 μg/ml in case of paclitaxel. Cells were allowed to attach overnight, then they were treated for 72 hours. At the end of the treatment period the cells were stained with MTT, after 4 hours solubilization solution was added. Formazan quantity was measured by a Multiscan FC spectrophotometer. Measurements were done in triplicates. IC_{50} values were determined by GraphPad Prism software. The cross-resistance of the cell lines was also determined, when doxorubicin, paclitaxel, 5-fluorouracil and cisplatin was used. The

clinical concentration was 64.65 μg/ml for 5-fluorouracil, and 14,3 μg/ml for cisplatin .

Establishment of resistant cell lines

10 cell lines were separated for each cell line and each drug. Cell lines were treated with doxorubicin or paclitaxel, concentration of treatment was gradually increased. If confluence was under 50% after 1 week of treatment, treatment was stopped, if it was between 50 and 70 % treatment was continued, if it was over 70% treatment was continued, and a section of the cells was frozen. If the cell lines grew for at least 3 weeks without treatment suspension, the concentration of the drug was increased. Before increasing the concentration, the MTT cell proliferation assay was used to confirm the sensitivity of the cell line.

Nucleic acid isolation

Homogenization of the samples was performed by Qiashredder, RNA isolation was performed by Qiagen RNEasy Mini kit according to the users manual. RNA quality and quantity was determined by a Nanodrop 1000 device. RNA was stored on -86 °C.

RT-PCR

TaqMan real time PCR (Micro Fluidic Card system, Applied Biosystems) was used to determine the mRNA expression of the selected genes in the resistant and parental cell lines. Measurements were done by a ABI PRISM® 7900HT Sequence Detection system according to the users manual. Expression of genes with correation to doxorubicin or paclitaxel resistance was measured. mRNA expression of hormone receptors, and genes associated with prognosis were also measured.

Cytogenetics

Control and treated MDA-MB-231 cell lines were kept in culture for 3-5 days, until 80% confluence was reached. Then, cells were treated with colcemid for 24 hours. After the colcemid treatment chromosome preparation was performed using a standard technique. Chromosome analysis was performed on metaphase cells G-banded with trypsin and Wright Giemsa stain. Ten metaphases were evaluated for each sample with an Axioskop 2 Mot Plus microscope and Cytovision 3.6 or Mac Ktype 5.6 computer analysis system for karyotyping. Shannon diversity index was computed to characterize the extent of genetic diversity and genetic instability based on the cytogenetic changes of the cell lines.

Flow cytometry

The P-glycoprotein activity was measured by the accumulation of the Pgp substrate rhodamin 123. For 30 minutes, 5 x 10^5 cells were treated with rhodamine 123, then cells were collected, and washed with ice cold PBS. Flow cytometric analysis determined the rhodamine 123 intensity in 10^4 cells.

Results

Establishment of resistant cell lines, results of cross resistance measurements

After the 18 months of the treatment, 29 cell lines became resistant (10 doxorubicin and 4 paclitaxel treated MCF7 and 6 doxorubicin and 9 paclitaxel treated MDA-MB-231). We determined the level of cross-resistance by determining the IC50 values against four widely used agents in the treatment of cancer (doxorubicin, paclitaxel, cisplatin and 5-fluorouracil). Although some of the cell lines exhibited significantly increased resistance against other agents as well, no significant correlation between the relative resistance levels was observed. The least degree of cross-resistance was observed in case of cisplatin treatment. Among the resistant cell lines four showed highly increased IC50 values in case of 5-fluorouracil treatment.

RT-PCR

Gene expression of a set of selected genes was measured by TaqMan real-time PCR. The gene expression levels were correlated to the IC50 values using Spearman's rank correlation test. In the doxorubicin resistant cell lines the expression of TOP2A (p=0.003) and two tubulin isoforms (TUBB2C, p=0.003; TUBB3, p=0.006) was correlated to the level of resistance significantly. In the paclitaxel resistant cell lines, the expression of MVP (p=0.009), of four tubulin isoforms (TUBA1C, p=0.003; TUBB2A, p=0.009, TUBB4, p=0.005) and of MAP4 (p=0.001) correlated to resistance. ABCB1 reached a p value of 0.03 and 0.07 in the doxorubicin- and pacalitaxel-treated cell lines, respectively, but these were not significant after multiple testing corrections.

Citogenetika

Cytogenetics was performed on all cell lines derived from the MDA-MB-231 cell line. There was a high number of genetic changes in the generated cell lines. The new cell lines had 60-110 chromosomes, chromosomes 1, 17 and 21 had the most copies. Based on G-band staining, the highest variability among the chromosomes were observed on chromosomes 3, 7, 17, 20 and 21. Most stable chromosomes were X, 10, 13 and 16. The most common gains were on chromosomes 15, 18 and 21. The most common deletions were 9p21 and 18q21. Two of the

cell lines (MDA-MB-231-R5 and MDA-MB-231-R11) differed from the other cell lines by having a nearly tetraploid modal chromosome number. The main difference between the parental and the developed cell lines is in the lower number of chromosomes with higher number of new type structural rearrangements in the derivative cell lines.

Chromosomal instability

The average Shannon index across all cell lines was 2.03, the parental MDA-MB-231 cell line had an index value of 2.05. Chromosomal instability (CIN+) was found in five cell lines. The CIN+ cell lines had a higher average cross-resistance as compared to the CIN- cell lines (for paclitaxel 7.1 vs. 6.7 μg/ml, cisplatin 4.2 vs. 3.5 μg/ml and 5-fluorouracil 10.1 vs. 5.1 μg/ml), but the differences between the two groups were not significant.

Flow cytometry

The ability of the cell lines to export drugs via Pgp-activity was assessed by FACS using the Pgp substrate rhodamine 123. The originally paclitaxel-resistant MCF-7-R20 cell lines showed the highest cross-resistance against doxorubicin. The paclitaxel-resistant MDA-MB-231-R19 showed the highest resistance against paclitaxel and also

showed high rhodamine efflux. Two paclitaxel-resistant cell lines (MCF-6-R5 and MCF-7-R7) showed also increased resistance against doxorubicin and cisplatin. However, a general correlation between rhodamine efflux and multidrug resistance for other agents and other cell lines could not be observed. These results suggest the important role of Pgp in the formation of resistance in some of the cell lines, while in others the activity stays unchanged.

Conclusions

My aim was to investigate the acquired resistance evolving independently in numerous cell lines derived from the same parental cell line. My assumption was, that this model will be able to describe the acquired resistance better, as comparing a single original and resistant cell line pair.

Conclusions of the study:

1. **The use of multiple parallel developed resistant cell lines, derived from the same original cell lines is a suitable model to validate multiple resistance mechanisms.** Additionally, in case of paclitaxel resistant cell lines, we managed to identify multiple previously described resistance-mechanisms with this model,

while previous studies commonly described only one resistance-mechanism.

2. **Evolution of multidrug resistance is a rare event after treatment with a single agent.**. Only 2 cell lines out of 29 showed at least twofold cross-resistance against 3 other drugs investigated.

3. **Resistance is heterogeneous in the resistant cell lines.** Flow cytometry measurements showed in some of the resistant cell lines a changed transport pump activity leading to resistance, while in other resistant cell lines the activity did not change. In case of the latter group other mechanisms led to the acquired resistance. TaqMan measurements showed similar results for other resistance mechanisms.

List of publications

Publications related to theses

1. Tegze B, Szállási Z, Haltrich I., Pénzváltó Z, Tóth Z, Likó I, Győrffy B: **Parallel evolution under chemotherapy pressure**

in 29 breast cancer cell lines results in dissimilar mechanisms of resistance. *PLoS ONE* 2012, 7(2):e30804. **IF:4.411**

2. Munkácsy G, Abdul-Ghani R, Mihály Z, Tegze B, Tchernitsa O, Surowiak P, Schäfer R, Györffy B: **PSMB7 is associated with anthracycline resistance and is a prognostic biomarker in breast cancer.** *Br J Cancer.* 2010, 102(2):361-8. **IF: 4.831**

Publications not related to the thesis

1. Fekete T, Rásó E, Pete I, Tegze B, Liko I, Munkácsy Gy, Sipos N, Rigó J. jr., Győrffy B: **Meta-analysis of gene expression profiles associated with histological classification and survival in 829 ovarian cancer samples.** *Int J Cancer.2011, accepted for publication,* **IF: 4.926**

2. Tegze B, Tulassay Z, Győrffy B: **Chemotherapy agents, response rates and mechanisms of resistance in the therapy of the colorectal carcinoma.** *Magy Onkol.* 2006;50(4):315-23.

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